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NOTES

Normal IncA Expression and Fusogenicity of Inclusions in *Chlamydia trachomatis* Isolates with the *incA* I47T Mutation

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To investigate the correlation between the *incA* I47T mutation in *Chlamydia trachomatis* and the nonfusogenic phenotype, the *incA* genes of 25 isolates were sequenced. Four major sequence types were identified. Seven isolates (28%) had the I47T mutation. Isolates representing the four sequence types expressed IncA in the membrane of one large single inclusion. In conclusion, the *incA* I47T mutation is not associated with the nonfusogenic phenotype.

Chlamydia trachomatis is an obligate intracellular pathogen that replicates inside human epithelial cells in a membrane-bound vacuole termed an inclusion (4, 16). The *C. trachomatis* developmental cycle in the infected cells is characterized by the fusion of various small homotypic inclusions containing reticulate bodies to one large single inclusion which then contains the replicating bacteria (5, 7). Key features for the survival of *C. trachomatis* in the cell and the pathogenesis of the *C. trachomatis* infection are the bacterial ability to prevent a fusion between this single inclusion and the cellular lysosomal compartments and the bacterial ability to acquire nutrients from the host cell cytosol (4, 16). Chlamydial proteins that are translocated into the inclusion membrane, so-called Inc proteins, might be involved in both processes (11). To date, at least 11 distinct Inc proteins in the inclusion membrane of *C. trachomatis*-infected cells have been identified (2, 3, 13). Although the various Inc proteins of *C. trachomatis* share a very low primary sequence homology, a typical bilobed hydrophobic domain of approximately 60 amino acids is present in all of them (2). Recently, *C. trachomatis* strains causing unusual nonfusogenic inclusions were identified (15). The growth cycle of this phenotype in epithelial cells was characterized by the occurrence of multiple small nonfusogenic inclusions in epithelial cells. This phenotype was present in only 176 (1.5%) of 11,440 clinical isolates (15). Immunofluorescence microscopy of cells infected with *C. trachomatis* of the nonfusogenic phenotype revealed that IncA was undetectable in the inclusion membrane. In addition, IncA could not be detected by immunoblot analysis in whole-cell lysates of cells infected with nonfusogenic *C. trachomatis* (15). These data strongly suggested that one of the functions of IncA is to facilitate homotypic fusion of inclusions. Sequence analysis of *incA* of two strains exhibiting

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TABLE 1. Alignment of polymorphic positions of deduced amino acid sequences of IncA[a]

Serovar/strain[b]	Polymorphic amino acid positions[c]	Sequence type	Accession no.
A/Sa-1	IAILEHGRQQVVKCSS	*a*	AF326992
Ba/Apache-2	....	*a*	AF326994
D-/NL-326	....	*a*	AF326998
E/2b	....	*a*	AF327012
E/10a	....	*a*	AF327014
E/11a	....	*a*	AF327015
I′	....	*a*	AF327004
J/UW-36	....	*a*	AF327006
G/IOL-238	....	*a*	AF327002
E/DK-20	T...............	*b*	AF327000
E/4a	T...............	*b*	AF327011
E/7a	T...............	*b*	AF327016
E/12a	T...............	*b*	AF327013
F/MRC-301	T...............	*b*	AF327001
D/IC-Cal-8	T...K...........	*c*	AF326996
D′	T...K...........	*c*	AF326999
H/UW-4	.T..............	*d*	AF327003
L2/434-B	..T......EAA....	*e*	AF327009
L1/440-L	..T...SCREAA....	*f*	AF327008
B/TW-5	..T...S.REAA....	*g*	AF326993
L3/404-L	..T...S.REAAR...	*h*	AF327010
C/UW-1	...P.........R..	*i*	AF326995
Da/MT-566	.....LD.........	*j*	AF326997
I/UW-12	...............F	*k*	AF327005
K/UW-31	..............P.	*l*	AF327007

[a] Underlined amino acid positions are located in the hydrophobic domain of IncA. The prototypic amino acid sequence deduced from the DNA sequence of strain D/UW-3/Cx (14) is indicated as sequence type a. A dot indicates amino acid identity with sequence type a. The amino acid sequence identical to that of the described nonfusogenic strains that do not express IncA (15) is indicated as sequence type *c*. Isolates in boldface type were tested for IncA expression and fusogenicity by immunofluorescence microscopy.

[b] The *Chlamydia* isolates were obtained from previously described sources (8, 9).

[c] The amino acids indicated for sequence type *a* correspond to positions I-49, A-62, I-75, L-90, E-116, H-119, G-180, R-200, Q-206, Q-207, V-212, V-213, K-235, C-252, S-260, and S-261.

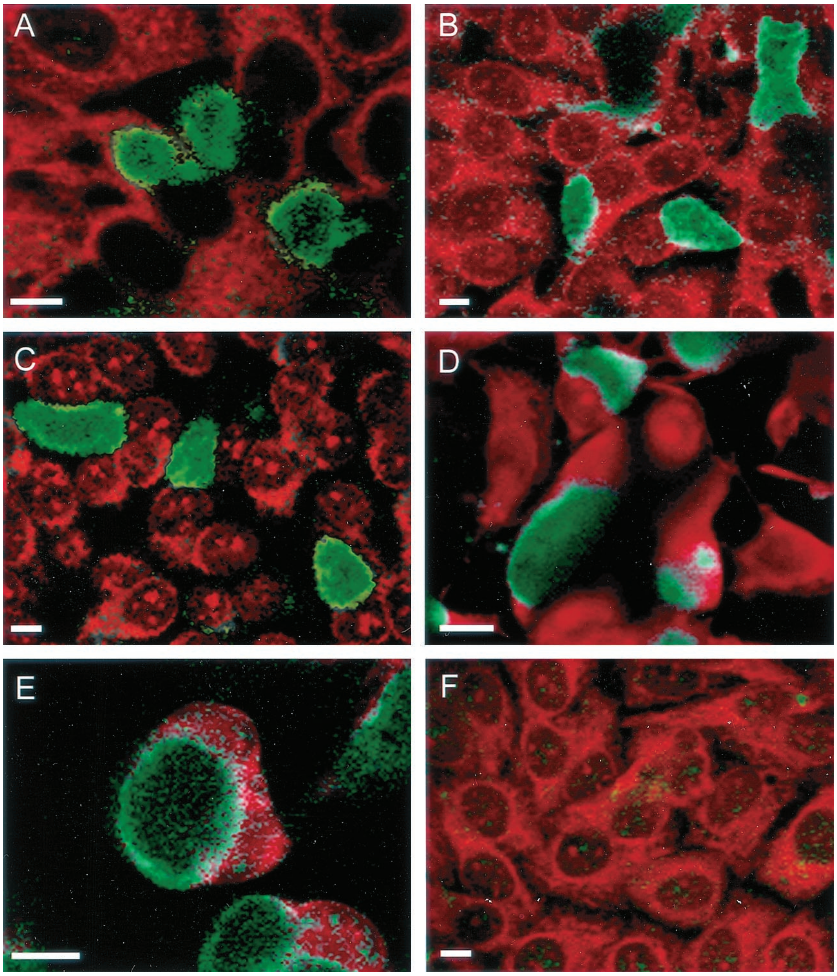

FIG. 1. Fluorescence microscopic analyses of HeLa cells infected with serovar D′, D, F, E, and L2 (panels A to E, respectively) and noninfected cells (F) fixed for microscopy 20 to 30 h postinfection. Immunostaining with anti-IncA indicates *incA* expression and the occurrence of fusogenicity in all infected cells, despite the presence of the mutations I47T and E116K (A and B), the mutation I47T (C and D), or any other polymorphisms in *incA* (E). The bar in each panel indicates 10 μm.

the nonfusogenic phenotype and lacking detectable IncA protein identified two nonsynonomous mutations in the gene compared to the DNA sequence of the prototypic *C. trachomatis* strain (serovar D/UW-3/Cx) (14). One mutation leads to the replacement of isoleucine at codon 47 with threonine (I47T), modifying the characteristic hydrophobic domain of the protein. The other substitution (E116K) is localized outside this domain (15). These results suggested that the *incA* I47T mutations might be associated with the nonfusogenic phenotype.

The present study aimed to investigate the correlation between the *incA* I47T mutation and the nonfusogenic phenotype in *C. trachomatis*. In order to assess the occurrence of the I47T polymorphism as well as possible additional polymorphisms in *incA*, the *incA* genes of 25 *C. trachomatis* isolates of different serovars were sequenced (Table 1). Selection of the isolates was at random, with the only condition being that all major serovars should be present. Isolates were propagated and DNA was extracted from infected cells as described previously (8). *incA* was then amplified by thermocycling using the primer pair 5′-AGCCATAGGATCTGGTTTCAGCGA-3′ and 5′-GCGCGGATCCTAGGAGCTTTTTGTAGAGGGTGA-3 (15). From each isolate, three independently generated PCR products, one of which was cloned into the PCR2.1 vector (Invitrogen, Groningen, The Netherlands), were sequenced using the above-mentioned primers, a second primer set (5′-CGCTCCACAAATCACTATTGTTC-3′ and 5′-TGAGGCAACAGAGCCTTTAAGA-3′), and -21M13 and M13Reverse primers (Perkin-Elmer, Nieuwekerk aan de IJssel, The Netherlands). Sequence analysis was performed on a automatic sequencer (Applied Biosystems Incorporated, Foster City, Calif.) as described previously (10). The accession numbers of *incA* sequences are listed in Table 1.

Database similarity searches (1) revealed that 19 out of 25 *incA* genes contained one or more point mutations compared to the DNA sequence of the prototypic *incA*-positive and fusogenic strain (D/UW-3/Cx) (14). No deletions were found, and none of the mutations introduced a stop codon.

On the basis of amino acid substitution patterns in the hydrophobic domain of IncA, there were four major different *incA* sequence types among the isolates. Nine (36%) of the strains studied contained *incA* genes encoding putative IncA proteins which were identical to the prototype sequence, indicated as sequence type a (Table 1). Four (16%) strains had *incA* sequences in which only substitutions outside the hydrophobic domain were identified (sequence types *i* to *l*) (Table 1). *incA* sequences from five (20%) other strains (sequence types *d* to *h*) were characterized by the presence of a mutation at codon 62 or at codon 75. Hydropathy plot analysis, using a window size of seven (6), predicted that none of these substitutions would result in a significant change in the secondary structure of the hydrophobic domain of IncA (not shown). In addition, it has been reported that such strains express IncA in the inclusion membrane and are fusogenic (3), indicating that the substitutions do not interfere with IncA expression and fusogenicity.

Seven (28%) strains with the mutation I47T (sequence types *b* and *c*) were identified. The frequency of this mutation was significantly higher than that described for the nonfusogenic phenotype (15). Therefore, it is questionable whether I47T predicts nonfusogenicity of strains carrying this mutation; thus, we studied IncA expression and fusogenicity of nine *Chlamydia* isolates representing the four *incA* sequence types (indicated in Table 1) using immunofluorescence microscopy. HeLa 229 cells (ATCC CCL2.1) cultured on coverslips were infected with each of the isolates a multiplicity of infection of 1 to 2. Infections were carried out as described previously (8), and cells were ethanol fixed 20 to 30 h postinfection and indirectly immunostained with an antiserum directed against IncA (3) essentially as described previously (12). Confocal immunofluorescence microscopy of infected cells immunostained with anti-IncA demonstrated clear bright fluorescent staining of one large single inclusion in each infected cell, irrespective of the sequence type of the strain used for infection (Fig. 1A to E). In addition, staining of reticulate bodies circumscribing the luminal surface of the single inclusions was also observed (Fig. 1A to E). In contrast, mock-infected cells showed no reactivity (Fig. 1F). The typical rim-like staining pattern of the inclusion membrane strongly indicates that the strains expressed IncA which was localized in the inclusion membrane. In addition, only one large inclusion per infected cell was present, indicating that the neither the I47T mutation nor one of the other *incA* polymorphisms interfered with the characteristically homotypic fusion of the inclusions. In conclusion, the alteration in the hydrophobic domain of IncA due to the mutation I47T is not associated with a deficiency in IncA expression and does not interfere with the translocation of IncA to the inclusion membrane and the fusogenicity of the inclusion. Therefore, other mutations in *incA* or its putative regulatory regions, alone or in conjunction with mutations and lesions in other genes, should be considered as alternative explanations for nonfusogenicity of inclusions.

Nucleotide sequence accession numbers. The nucleotide sequences of *incA* are deposited in the GenBank database under the accession numbers indicated in Table 1.

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REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Bannantine, J. P., R. S. Griffiths, W. Viratyosin, W. J. Brown and D. D. Rockey.** 2000. A secondary structure motif predictive of protein localization to the chlamydial inclusion membrane. Cell. Microbiol. **2:**35–47.
3. **Bannantine, J. P., W. E. Stamm, R. J. Suchland, and D. D. Rockey.** 1998. *Chlamydia trachomatis* IncA is localized to the inclusion membrane and is recognized by antisera from infected humans and primates. Infect. Immun. **66:**6017–6021.
4. **Hackstadt, T., E. R. Fischer, M. A. Scidmore, D. D. Rockey, and R. A. Heinzen.** 1997. Origins and functions of the chlamydial inclusion. Trends Microbiol. **5:**288–293.
5. **Hodinka, R. L., C. H. Davis, J. Choong, and P. B. Wyrick.** 1988. Ultrastructural study of endocytosis of *Chlamydia trachomatis* by McCoy cells. Infect. Immun. **56:**1456–1463.
6. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
7. **Matsumoto, A., H. Bessho, K. Uehira, and T. Suda.** 1991. Morphological studies of the association of mitochondria with chlamydial inclusions and the fusion of chlamydial inclusions. J. Electron Microsc. **40:**356–363.
8. **Meijer, A., S. A. Morré, A. J. C. van den Brule, P. H. M. Savelkoul, and J. M. Ossewaarde.** 1999. Genomic relatedness of *Chlamydia* isolates determined by amplified fragment length polymorphism analysis. J. Bacteriol. **181:**4469–4475.
9. **Morré, S. A., J. M. Ossewaarde, P. H. M. Savelkoul, J. Stoof, C. J. L. M. Meijer, and A. J. C. van den Brule.** 2000. Analysis of genetic heterogeneity in *Chlamydia trachomatis* clinical isolates of serovars D, E, and F by amplified fragment length polymorphism. J. Clin. Microbiol. **38:**3463–3466.
10. **Pannekoek, Y., S. M. Westenberg, J. de Vries, S. Repping, L. Spanjaard, P. P. Eijk, A. van der Ende, and J. Dankert.** 2000. PCR assessment of *Chlamydia trachomatis* infection of semen specimens processed for artificial insemination. J. Clin. Microbiol. **38:**3763–3767.
11. **Rockey, D. D., J. Lenart, and R. S. Stephens.** 2000. Genome sequencing and our understanding of chlamydiae. Infect. Immun. **68:**5473–5479.
12. **Rockey, D. D., R. A. Heinzen, and T. Hackstadt.** 1995. Cloning and characterization of a *Chlamydia psittaci* gene coding for a protein localized in the inclusion membrane of infected cells. Mol. Microbiol. **15:**617–626.
13. **Scidmore-Carlson, M. A., E. I. Shaw, C. A. Dooley, E. R. Fischer, and T. Hackstadt.** 1999. Identification and characterization of a *Chlamydia trachomatis* early operon encoding four novel inclusion membrane proteins. Mol. Microbiol. **33:**753–765.
14. **Stephens, R. S., S. Kalman, C. Lammel, J. Fan, R. Marathe, L. Aravind, W. Mitchell, L. Olinger, R. L. Tatusov, Q. Zhao, E. V. Koonin, and R. W. Davis.** 1998. Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. Science **282:**754–759.
15. **Suchland, R. J., D. D. Rockey, J. P. Bannantine, and W. E. Stamm.** 2000. Isolates of *Chlamydia trachomatis* that occupy nonfusogenic inclusions lack IncA, a protein localized to the inclusion membrane. Infect. Immun. **68:**360–367.
16. **Wyrick, P. B.** 2000. Intracellular survival by *Chlamydia*. Cell. Microbiol. **2:**275–282.